ANALYSING POLYNUCLEOTIDE SEQUENCES

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1. INTRODUCTION

Three methods dominate molecular analysis of
nucleic acid sequences: gel electrophoresis of
restriction fragments, molecular hybridisation, and the
rapid DNA sequencing methods. These three methods have

5 a very wide range of applications in biology, both in
basic studies, and in the applied areas of the subject
such as medicine and agriculture. Some idea of the
scale on which the methods are now used is given by the
rate of accumulation of DNA sequences, which is now

10 well over one million base pairs a year. However,
powerful as they are, they have their limitations. The
restriction fragment and hybridisation methods give a
coarse analysis of an extensive region, but are rapid;
sequence analysis gives the ultimate resolution, but it

15 is slow, analysing only a short stretch at a time.
There is a need for methods which are faster than the
present methods, and in particular for methods which
cover a large amount of sequence in each analysis.

This invention provides a new approach which

20 produces both a fingerprint and a partial or complete
sequence in a single analysis, and may be used directly
with complex DNAs and populations of RNA without the
need for cloning.

In one aspect the invention provides

25 apparatus for analysing a polynucleotide sequence,
comprising a support and attached to a surface therof
an array of the whole or a chosen part of a complete
set of oligo nucleotides of chosen lengths, the
different oligonucleotides occupying separate cells of

30 the array and being capable of taking part in
hybridisation reactions. For studying differences
between polynucleotide sequences, the invention
provides in another aspect apparatus comprising a
support and attached to a surface therof an array of

35 the whole or a chosen part of a complete set of
oligonucleotides of chosen lengths comprising the
polynucleotide sequences, the different

oligonucleotides occupying separate cells of the array and being capable of taking part in hybridisation reactions.

In another aspect, the invention provides a method of analysing a polynucleotide sequence, by the use of a support to the surface of which is attached an array of the whole or a chosen part of a complete set of oligo nucleotides of chosen lengths, the different oligonucleotides occupying separate cells of the array, which method comprises labelling the polynucleotide sequence or fragments thereof to form labelled material, applying the labelled material under hybridisation conditions to the array, and observing the location of the label on the surface associated with particular members of the set of oligonucleotides.

The idea of the invention is thus to provide a structured array of the whole or a chosen part of a complete set of oligonucleotides of one or several chosen lengths. The array, which may be laid out on a supporting film or glass plate, forms the target for a hybridisation reaction. The chosen conditions of hybridisation and the length of the oligonucleotides must at all events be sufficient for the available equipment to be able to discriminate between exactly matched and mismatched oligonucleotides. In the hybridisation reaction, the array is explored by a labelled probe, which may comprise oligomers of the chosen length or longer polynucleotide sequences or fragments, and whose nature depends on the particular application. For example, the probe may comprise labelled sequences amplified from genomic DNA by the polymerase chain reaction, or a mRNA population, or a complete set of oligonucleotides from a complex sequence such as an entire genome. The end result is a set of filled cells corresponding to the oligonucleotides present in the analysed sequence, and a set of "empty" sites corresponding to the sequences

which are absent in the analysed sequence. The pattern produces a fingerprint representing all of the sequence analysed. In addition, it is possible to assemble most or all of the sequence analysed if an oligonucleotide

5   length is chosen such that most or all oligonucleotide sequences occur only once.

The number, the length and the sequences of the oligonucleotides present in the array "lookup table" also depend on the application. The array may include

10   all possible oligonucleotides of the chosen length, as would be required if there was no sequence information on the sequence to be analysed. In this case, the preferred length of oligonucleotide used depends on the length of the sequence to be analysed, and is such that

15   there is likely to be only one copy of any particular oligomer in the sequence to be analysed. Such arrays are large. If there is any information available on the sequence to be analysed, the array may be a selected subset. For the analysis of a sequence which

20   is known, the size of the array is of the same order as length of the sequence, and for many applications, such as the analysis of a gene for mutations, it can be quite small. These factors are discussed in detail in what follows.

25   2.   OLIGONUCLEOTIDES AS SEQUENCE PROBES

Oligonucleotides form base paired duplexes with oligonucleotides which have the complementary base sequence. The stability of the duplex is dependent on the length of the oligonucleotides and on base

30   composition. Effects of base composition on duplex stability can be greatly reduced by the presence of high concentrations of quaternary or tertiary amines. However, there is a strong effect of mismatches in the oligonucleotides duplex on the thermal stability of the

35   hybrid, and it is this which makes the technique of

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hybridisation with oligonucleotides such a powerful
method for the analysis of mutations, and for the
selection of specific sequences for amplification by
DNA polymerase chain reaction. The position of the
5   mismatch affects the degree of destabilisation.
Mismatches in the centre of the duplex may cause a
lowering of the Tm by 10°C compared with 1°C for a
terminal mismatch. There is then a range of
discriminating power depending on the position of
10   mismatch, which has implications for the method
described here. There are ways of improving the
discriminating power, for example by carrying out
hybridisation close to the Tm of the duplex to reduce
the rate of formation of mismatched duplexes, and by
15   increasing the length of oligonucleotide beyond what is
required for unique representation. A way of doing
this systematically is discussed.
3.   ANALYSIS OF A PREDETERMINED SEQUENCE
     One of the most powerful uses of oligonucleotide
20   probes has been in the detection of single base changes
in human genes. The first example was the detection of
the single base change in the betaglobin gene which
leads to sickle cell disease. There is a need to
extend this approach to genes in which there may be a
25   number of different mutations leading to the same
phenotype, for example the DMD gene and the HPRT gene,
and to find an efficient way of scanning the human
genome for mutations in regions which have been shown
by linkage analysis to contain a disease locus for
30   example Huntington's disease and Cystic Fibrosis. Any
known sequence can be represented completely as a set
of overlapping oligonucleotides. The size of the set
is N s + 1 $\approx$ N, where N is the length of the sequence
and s is the length of an oligomer. A gene of 1 kb for
35   example, may be divided into an overlapping set of

around one thousand oligonucleotides of any chosen
length. An array constructed with each of these
oligonucleotides in a separate cell can be used as a
multiple hybridisation probe to examine the homologous
5   sequence in any context, a single-copy gene in the human
genome or a messenger RNA among a mixed RNA population,
for example. The length s may be chosen such that
there is only a small probability that any oligomer in
the sequence is represented elsewhere in the sequence
10 to be analysed. This can be estimated from the
expression given in the section discussing statistics
below. For a less complete analysis it would be
possible to reduce the size of the array e.g. by a
factor of up to 5 by representing the sequence in a
15 partly or non-overlapping set. The advantage of using
a completely overlapping set is that it provides a more
precise location of any sequence difference, as the
mismatch will scan in s consecutive oligonucleotides.
4.   ANALYSIS OF AN UNDETERMINED SEQUENCE
20   The genomes of all free living organisms are
larger than a million base pairs and none has yet been
sequenced completely. Restriction site mapping reveals
only a small part of the sequence, and can detect only
a small portion of mutations when used to compare two
25 genomes. More efficient methods for analysing complex
sequences are needed to bring the full power of
molecular genetics to bear on the many biological
problems for which there is no direct access to the
gene or genes involved. In many cases, the full
30 sequence of the nucleic acids need not be determined;
the important sequences are those which differ between
two nucleic acids. To give three examples: the DNA
sequences which are different between a wild type
organism and one which carries a mutant can lead the
35 way to isolation of the relevant gene; similarly, the
sequence differences between a cancer cell and its

normal counterpart can reveal the cause of transfor-
mation; and the RNA sequences which differ between two
cell types point to the functions which distinguish
them. These problems can be opened to molecular
5 analysis by a method which identifies sequence
differences. Using the approach outlined here, such
differences can be revealed by hybridising the two
nucleic acids, for example the genomic DNA of the two
genotypes, or the mRNA populations of two cell types to
10 an array of oligonucleotides which represent all
possible sequences. Positions in the array which are
occupied by one sequence but not by the other show
differences in two sequences. This gives the sequence
information needed to synthesise probes which can then
15 be used to isolate clones of the sequence involved.
4.1 ASSEMBLING THE SEQUENCE INFORMATION
      Sequences can be reconstructed by examining the
result of hybridisation to an array. Any oligonucleo-
tide of length s from within a long sequence, overlaps
20 with two others over a length s-1. Starting from each
positive oligonucleotide, the array may be examined for
the four oligonucleotides to the left and the four to
the right that can overlap with a one base displacement.
If only one of these four oligonucleotides is found to
25 be positive to the right, then the overlap and the
additional base to the right determine s bases in the
unknown sequence. The process is repeated in both
directions, seeking unique matches with other positive
oligonucleotides in the array. Each unique match adds
30 a base to the reconstructed sequence.
4.2 SOME STATISTICS
      Any sequence of length N can be broken down to a
set of ~ N overlapping sequences s base pairs in
length. (For double stranded nucleic acids, the
35 sequence complexity of a sequence of N base pairs is
2N, because the two strands have different sequences.

but for the present purpose. this factor of two is not
significant). For oligonucleotides of length s, there
are 4^s different sequence combinations. How big should
s be to ensure that most oligonucleotides will be
represented only once in the sequence to be analysed,
of complexity N? For a random sequence the expected
number of s-mers which will be present in more than one
copy is

$$\mu_{>1} \approx 4^s(1 - e^{-\lambda}(1 + \lambda))$$

where

$$\lambda = (N - s + 1)/4^s$$

For practical reasons it is also useful to know
how many sequences are related to any given s-mer by a
single base change. Each position can be substituted
by one of three bases, there are therefore 3s sequences
related to an individual s-mer by a single base change,
and the probability that any s-mer in a sequence of N
bases is related to any other s-mer in that sequence
allowing one substitution is 3s x $N/4^s$. The relative
signals of matched and mismatched sequences will then
depend on how good the hybridisation conditions are in
distinguishing a perfect match from one which differ by
a single base. (If 4^s is an order of magnitude greater
than N, there should only be a few, 3s/10, related to
any oligonucleotide by one base change.) The
indications are that the yield of hybrid from the mis-
matched sequence is a fraction of that formed by the
perfect duplex.

For what follows, it is assumed that conditions
can be found which allow oligonucleotides which have
complements in the probe to be distinguished from those
which do not.

4.3 ARRAY FORMAT, CONSTRUCTION AND SIZE

To form an idea of the scale of the arrays needed

to analyse sequences of different complexity it is
convenient to think of the array as a square matrix.
All sequences of a given length can be represented just
once in a matrix constructed by drawing four rows

5 representing the four bases, followed by four similar
columns. This produces a 4 x 4 matrix in which each of
the 16 squares represents one of the 16 doublets. Four
similar matrices, but one quarter the size, are then
drawn within each of the original squares. This

10 produces a 16 x 16 matrix containing all 256 tetra-
nucleotide sequences. Repeating this process produces
a matrix of any chosen depth, s, with a number of cells
equal to 4^s. As discussed above, the choice of s is of
great importance, as it determines the complexity of

15 the sequence representation. As discussed below, s
also determines the size of the matrix constructed,
which must be very big for complex genomes. Finally,
the length of the oligonucleotides determines the
hybridisation conditions and their discriminating power

20 as hybridisation probes.

s	4^s	Genomes $4^s \times 10$	Side of Matrix (pixel=100 µm)	Number of Sheets of film
8	65536			
9	262144			
10	1.0×10^6	cosmid	100 mm	1
11	4.2×10^6			
12	1.7×10^7			
13	6.7×10^7	E.coli		
14	2.6×10^8	yeast	1.6 m	9
15	1.1×10^9			
16	4.2×10^9			
17	1.7×10^{10}			
18	6.7×10^{10}	human	25 m	2,500
19	2.7×10^{11}			
20	1.1×10^{12}		100 m	

The table shows the expected scale of the arrays
needed to perform the first analysis of a few genomes.
The examples were chosen because they are genomes which
have either been sequenced by conventional procedures -
5   the cosmid scale -, are in the process of being
sequenced - the E. coli scale -, or for which there has been
considerable discussion of the magnitude of the problem
- the human scale. the table shows that the expected
scale of the matrix approach is only a small fraction
10   of the conventional approach. This is readily seen in
the area of X-ray film that would be consumed. It is
also evident that the time taken for the analysis would
be only a small fraction of that needed for gel
methods. The "Genomes" column shows the length of
15   random sequence which would fill about 5% of cells in
the matrix. This has been determined to be the optimum
condition for the first step in the sequencing strategy
discussed below. At this size, a high proportion of
the positive signals would represent single occurrences
20   of each oligomer, the conditions needed to compare two
genomes for sequence differences.
5.   REFINEMENT OF AN INCOMPLETE SEQUENCE
Reconstruction of a complex sequence produces a
result in which the reconstructed sequence is
25   interrupted at any point where an oligomer that is
repeated in the sequence occurs. Some repeats are
present as components of long repeating structures
which form part of the structural organisation of the
DNA, dispersed and tandum repeats in human DNA for
30   example. But when the length of oligonucleotide used
in the matrix is smaller than that needed to give
totally unique sequence representation, repeats occur
by chance. Such repeats are likely to be isolated.
That is, the sequences surrounding the repeated
35   oligomers are unrelated to each other. The gaps caused

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by these repeats can be removed by extending the
sequence to longer oligomers. In principle, those
sequences shown to be repeated by the first analysis.
using an array representation of all possible
5   oligomers, could be resynthesised with an extension at
each end. For each repeated oligomer, there would be 4
x 4 = 16 oligomers in the new matrix. The
hybridisation analysis would now be repeated until the
sequence was complete. In practice, because the
10 results of a positive signal in the hybridisation may
be ambiguous, it may be better to adopt a refinement of
the first result by extending all sequences which did
not give a clear negative result in the first analysis.
An advantage of this approach is that extending the
15 sequence brings mismatches which are close to the ends
in the shorter oligomer, closer to the centre in the
extended oligomer, increasing the discriminatory power
of duplex formation.
5.1   A HYPOTHETICAL ANALYSIS OF THE SEQUENCE OF
20   BACTERIOPHAGE λ DNA
        Lambda phage DNA is 48,502 base pairs long. Its
sequence has been completely determined, we have
treated one strand of this as a test case in a computer
simulation of the analysis. The table shows that the
25 appropriate size of oligomer to use for a sequence of
this complexity is the 10-mer. With a matrix of 10-
mers, the size was 1024 lines square. After
"hybridisation" of the lambda 10-mers in the computer,
46,377 cells were positive, 1957 had double
30 occurrences, 75 triple occurrences, and three quadruple
occurrences. These 46,377 positive cells represented
known sequences, determined from their position in the
matrix. Each was extended by four x one base at the 3'
end and four x one base at the 5', end to give 16 x
35 46,377 = 742,032 cells. This extended set reduced the

number of double occurrences to 161, a further 16-
fold extension brought the number down to 10, and one
more provided a completely overlapped result. Of
course, the same end result of a fully overlapped

5   sequence could be achieved starting with a 4^{16} matrix,
but the matrix would be 4030 times bigger than the
matrix needed to represent all 10-mers, and most of the
sequence represented on it would be redundant.

5.2   LAYING DOWN THE MATRIX

10   The method described here envisages that the
matrix will be produced by synthesising oligonucleotides
in the cells of an array by laying down the precursors
for the four bases in a predetermined pattern, an
example of which is described above. Automatic

15   equipment for applying the precursors has yet to be
developed, but there are obvious possibilites; it
should not be difficult to adapt a pen plotter or other
computer-controlled printing device to the purpose.
The smaller the pixel size of the array the better, as

20   complex genomes need very large numbers of cells.
However, there are limits to how small these can be
made. 100 microns would be a fairly comfortable upper
limit, but could probably not be achieved on paper for
reasons of texture and diffusion. On a smooth

25   impermeable surface, such as glass, it may be possible
to achieve a resolution of around 10 microns, for
example by using a laser typesetter to preform a
solvent repellant grid, and building the oligonucleo-
tides in the exposed regions. One attractive

30   possibility, which allows adaptation of present
techniques of oligonucleotide synthesis, is to sinter
microporous glass in microscopic patches onto the
surface of a glass plate. Laying down very large
number of lines or dots could take a long time, if the

35   printing mechanism were slow. However, a low cost ink-

jet printer can print at speeds of about 10,000 spots per second. With this sort of speed, 10^8 spots could be printed in about three hours.

5   5.3   OLIGONUCLEOTIDE SYNTHESIS

There are several methods of synthesising oligonucleotides. Most methods in current use attach the nucleotides to a solid support of controlled pore size glass (CPG) and are suitable for adaptation to

10   synthesis on a glass surface. Although we know of no description of the direct use of oligonucleotides as hybridisation probes while still attached to the matrix on which they were synthesised, there are reports of the use of oligonucleotides as hybridisation probes on

15   solid supports to which they were attached after synthesis. PCT Application WO 85/01051 describes a method for synthesising oligonucleotides tethered to a CPG column. In an experiment performed by us, CPG was used as the support in an Applied Bio-sytems

20   oligonucleotide synthesiser to synthesise a 13-mer complementary to the left hand cos site of phage lambda. The coupling steps were all close to theoretical yield. The first base was stably attached to the support medium through all the synthesis and

25   deprotection steps by a covalent link.

5.4   ANALYSING SEVERAL SEQUENCES SIMULTANEOUSLY

The method of this invention can be used to analyse several polynucleotide sequences

30   simultaneously. To achieve this, the oligonucleotides may be attached to the support in the form of (for example) horizontal stripes. A technique for doing this is described in Example 3 below. Each DNA sample to be analysed is labelled and applied to the surface

35   carrying the oligonucleotides in the form of a stripe (e.g. vertical) orthogonal to the oligonucleotide

stripes of the array. Hybridisation is seen at the
intersections between oligonucleotide stripes and
stripes of test sequence where there is homology
between them.

5   Where sequence variations are known, an
advantage of using this technique is that many
different mutations can be probed simultaneously by
laying down stripes corresponding to each allelic
variant. With a density of one oligonucleotide per

10   mm, and one "individual" per 5 mm, it should be
possible to analyse 2000 loci on a plate 100 mm square.
Such a high density of information, where the
oligonucleotides do identify specific alleles, is not
available by other techniques.

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6.   PROBES, HYBRIDISATION AND DETECTION
       The yield of oligonucleotides synthesised on
microporous glass is about 30 μmol/g. A patch of this
material 1 micron thick by 10 microns square would hold

20   $\approx$ 3 x 10^{-12} μmol, equivalent to about 2 g of human

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DNA. The hybridisation reaction could therefore be carried out with a very large excess of the bound oligonucleotides over that in the probe. So it should be possible to design a system capable of

5 distinguishing between hybridisation involving single and multiple occurrances of the probe sequence, as yield will be proportional to concentration at all stages in the reaction.

The polynucleotide sequence to be analysed may be

10 of DNA or RNA. To prepare the probe, the polynucleotide may be degraded to form fragments. Preferably it is degraded by a method which is as random as possible, to an average length around the chosen length s of the oligonucleotides on the support, and oligomers of exact

15 length s selected by electrophoresis on a sequencing gel. The probe is then labelled. For example, oligonucleotides of length s may be end labelled. If labelled with ^{32}P, the radioactive yield of any individual s-mer even from total human DNA could be

20 more than 10^4 dpm/mg of total DNA. For detection, only a small fraction of this is needed in a patch 10-100 microns square. This allows hybridisation conditions to be chosen to be close to the Tm of duplexes, which decreases the yield of hybrid and decreases the rate of

25 formation, but increases the discriminating power. Since the bound oligonucleotide is in excess, signal need not be a problem even working close to equilibrium.

Hybridisation conditions can be chosen to be those

30 known to be suitable in standard procedures used to hybridise to filters, but establishing optimum conditions is important. In particular, temperature needs to be controlled closely, preferably to better than ±0.5°C. Particularly when the chosen length of

35 the oligonucleotide is small, the analysis needs to be

able to distinguish between slight differences of rate and/or extent of hybridisation. The equipment may need to be programmed for differences in base composition between different oligonucleotides. In constructing

5   the array, it may be preferable to partition this into sub-matrices with similar base compositions. This may make it easier to define the Tm which may differ slightly according to the base composition.

The choice of hybridisation solvent is

10   significant. When 1M NaCl is used, G:C base pairs are more stable than A:T base pairs. Double stranded oligonucleotides with a high G+Ccontent have a higher Tm than corresponding oligonucleotides with a high A+T content. This discrepancy can be compensated in

15   various ways: the amount of oligonucleotide laid down on the surface of the support can be varied depending on its nucleotide composition; or the computer used to analyse the data can be programmed to compensate for variations in nucleotide composition. A preferred

20   method, which can be used either instead of or in addition to those already mentioned, is to use a chaotropic hybridisation solvent, for example a quarternary or tertiary amine as mentioned above. Tetramethylammoniumchloride (TMACl) has proved

25   particularly suitable, at concentrations in the range 2 M to 5.5 M. At TMACl concentrations around 3.5 M to 4 M, the T_m dependence on nucleotide composition is greatly reduced.

The nature of the hybridisation salt used also has

30   a major effect on the overall hybridisation yield. Thus, the use of TMACl at concentrations up to 5 M can increase the overall hybridisation yield by a factor of 30 or more (the exact figure depending to some extent on nucleotide composition) in comparison with

35   hybridisation using 1M NaCl. Manifestly, this has important implications; for example the amount of

probe material that needs to be used to achieve a given
signal can be much lower.

Autoradiography, especially with ^{32}P causes image
degradation which may be a limiting factor determining

5   resolution; the limit for silver halide films is around
25 microns. Obviously some direct detection system
would be better. Fluorescent probes are envisaged;
given the high concentration of the target
oligonucleotides, the low sensitivity of fluorescence

10   may not be a problem.

We have considerable experience of scanning auto-
radiographic images with a digitising scanner. Our
present design is capable of resolution down to 25
microns, which could readily be extended down to less

15   than present application, depending on the quality of
the hybridisation reaction, and how good it is at
distinguishing absence of a sequence from the presence
of one or more. Devices for measuring astronomical
plates have an accuracy around 1 μ. Scan speeds are

20   such that a matrix of several million cells can be
scanned in a few minutes. Software for the analysis of
the data is straight-forward, though the large data
sets need a fast computer.

Experiments presented below demonstrate the

25   feasibility of the claims.

Commercially available microscope slides (BDH
Super Premium 76 x 26 x 1 mm) were used as supports.
These were derivatised with a long aliphatic linker
that can withstand the conditions used for the

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deprotection of the aromatic heterocyclic bases, i.e. 30% NH_3 at 55° for 10 hours. The linker, bearing a hydroxyl group which serves as a starting point for the subsequent oligonucleotide, is synthesised in two steps. The slides are first treated with a 25% solution of 3-glycidoxypropyltriethoxysilane in xylene containing several drops of Hunig's base as a catalyst. The reaction is carried out in a staining jar, fitted with a drying tube, for 20 hours at 90°C. The slides are washed with MeOH, Et_2O and air dried. Then neat hexaethylene glycol and a trace amount of conc. sulphuric acid are added and the mixture kept at 80° for 20 hours. The slides are washed with MeOH, Et_2O , air dried and stored desiccated at -20° until use. This preparative technique is described in British Patent Application 8822228.6 filed 21 September 1988.

The oligonucleotide synthesis cycle is performed as follows:

The coupling solution is made up fresh for each step by mixing 6 vol. of 0.5M tetrazole in anhydrous acetonitrile with 5 vol. of a 0.2M solution of the required beta-cyanoethylphosphoramidite. Coupling time is three minutes. Oxidation with a 0.1M solution of I_2 in THF/pyridine/H_2O yields a stable phospho-triester bond. Detritylation of the 5' end with 3% trichloroacetic acid in dichloromethane allows further extension of the oligonucleotide chain. There was no capping step since the excess of phosphoramidites used over reactive sites on the slide was large enough to drive the coupling to completion. After the synthesis is completed, the oligonucleotide is deprotected in 30% NH_3 for 10 hours at 55°. The chemicals used in the coupling step are moisture-sensitive, and this critical step must be performed under anhydrous conditions in a sealed container, as follows. The shape of the patch

to be synthesised was cut out of a sheet of silicone
rubber (76 x 26 x 0.5 mm) which was sandwiched between
a microscope slide, derivatised as described above, and
a piece of teflon of the same size and thickness.   To
5   this was fitted a short piece of plastic tubing that
allowed us to inject and withdraw the coupling solution
by syringe and to flush the cavity with Argon.   The
whole assembly was held together by fold-back paper
clips.   After coupling the set-up was disassembled and
10   the slide put through the subsequent chemical reactions
(oxidation with iodine, and detritylation by treatment
with TCA) by dipping it into staining jars.

EXAMPLE 1.

As a first example we synthesised the sequences
15   oligo-dT$_{10}$-oligo-dT$_{14}$ on a slide by gradually decreasing
the level of the coupling solution in steps 10 to 14.
Thus the 10-mer was synthesised on the upper part of
the slide, the 14-mer at the bottom and the 11, 12 and
13-mers were in between.   We used 10 pmol oligo-dA$_{12}$,
20   labelled at the 5' end with ^{32}P by the polynucleotide
kinase reaction to a total activity of 1.5 million
c.p.m., as a hybridisation probe.   Hybridisation was
carried out in a perspex (Plexiglas) container made to
fit a microscope slide, filled with 1.2 ml of 1M NaCl
25   in TE, 0.1% SDS, for 5 minutes at 20^0.   After a short
rinse in the same solution without oligonucleotide, we
were able to detect more than 2000 c.p.s. with a
radiation monitor.   An autoradiograph showed that all
the counts came from the area where the oligonucleotide
30   had been synthesised, i.e. there was no non-specific
binding to the glass or to the region that had been
derivatised with the linker only.   After partial
elution in 0.1 M NaCl differential binding to the
target is detectable, i.e. less binding to the shorter
35   than the longer oligo-dT.   By gradually heating the

slide in the wash solution we determined the T_{om} (mid-
point of transition when 50% eluted) to be 33°. There
were no counts detectable after incubation at 39°.
The hybridisation and melting was repeated eight times
with no diminution of the signal. The result is
reproducible. We estimate that at least 5% of the
input counts were taken up by the slide at each cycle.

EXAMPLE 2.

In order to determine whether we would be able to
distinguish between matched and mismatched oligo-
nucleotides we synthesised two sequences 3' CCC GCC GCT
GGA (cosL) and 3' CCC GCC TCT GGA, which differ by one
base at position 7. All bases except the seventh were
added in a rectangular patch. At the seventh base,
half of the rectangle was exposed in turn to add the
two different bases, in two stripes. Hybridisation of
cosR probe oligonucleotide (5' GGG CGG CGA CCT) (kinase
labelled with ^{32}P to 1.1 million c.p.m., 0.1 M NaCl,
TE, C.1% SDS) was for 5 hours at 32°. The front of the
slide showed 100 c.p.s. after rinsing. Autoradiography
showed that annealing occurred only to the part of the
slide with the fully complementary oligonucleotide.
No signal was detectable on the patch with the
mismatched sequence.

EXAMPLE 3.

For a further study of the effects of mismatches
or shorter sequences on hybridisation behaviour, we
constructed two arrays; one (a) of 24 oligonucleotides
and the other (b) of 72 oligonucleotides.

These arrays were set out as shown in Table 1(a)
and 1(b). The masks used to lay down these arrays were
different from those used in previous experiments.
Lengths of silicone rubber tubing (1mm o.d.) were glued
with silicone rubber cement to the surface of plain
microscope slides, in the form of a "U". Clamping

these masks against a derivatised microscope slide produced a cavity into which the coupling solution was introduced through a syringe. In this way only the part of the slide within the cavity came into contact with the phosphoramidite solution. Except in the positons of the mismatched bases, the arrays listed in Table 1 were laid down using a mask which covered most of the width of the slide. Off-setting this mask by 3mm up or down the derivatised slide in subsequent coupling reactions produced the olignucleotides truncated at the 3' or 5' ends.

For the introduction of mismatches a mask was used which covered half (for array (a)) or one third (for array (b)) of the width of the first mask. The bases at positions six and seven were laid down in two or three longitudinal stripes. This led to the synthesis of oligonucleotides differing by one base on each half (array (a)) or third (array (b)) of the slide. In other positions, the sequences differed from the longest sequence by the absence of bases at the ends.

In array (b), there were two columns of sequences between those shown in Table 1(b), in which the sixth and seventh bases were missing in all positions, because the slide was masked in a stripe by the silicone rubber seal. Thus there were a total of 72 different sequences represented on the slide in 90 different positions.

The 19-mer 5' CTC CTG AGG AGA AGT CTG C was used for hybridisation (2 million cpm, 1.2 ml 0.1M NaCl in TE, 0.1% SDS, 20°).

The washing and elution steps were followed by autoradiography. The slide was kept in the washing solution for 5 min at each elution step and then exposed (45 min, intensified). Elution temperatures were 23, 36, 42, 47, 55 and 60°C respectively.

As indicated in the table, the oligonucleotides
showed different melting behaviour.   Short oligo-
nucleotides melted before longer ones, and at 55°C,
only the perfectly matched 19-mer was stable, all other
5   oligonucleotides had been eluted.   Thus the method can
differentiate between a 18-mer and a 19-mer which
differ only by the absence of one base at the end.
Mismatches at the end of the oligonucleotides and at
internal sites can all be melted under conditions where
10 the perfect duplex remains.

Thus we are able to use very stringent
hybridisation conditions that eliminate annealing to
mismatch sequences or to oligonucleotides differing in
length by as little as one base.   No other method using
15 hybridisation of oligonucleotides bound to the solid
supports is so sensitive to the effects of mismatching.

EXAMPLE 4.

To test the application of the invention to
diagnosis of inherited diseases, we hybridised the
20 array (a), which carries the oligonucleotide sequences
specific for the wild type and the sickle cell mutations
of the β -globin gene, with a 110 base pair fragment of
DNA amplified from the β -globin gene by means of the
polymerase chain reaction (PCR).   Total DNA from the
25 blood of a normal individual (1 microgram) was
amplified by PCR in the presence of appropriate primer
oligonucleotides.   The resulting 110 base pair fragment
was purified by electrophoresis through an agarose gel.
After elution, a small sample (ca. 10 picogram) was
30 labelled by using α -^{32}P-dCTP (50 microCurie) in a
second PCR reaction.   This PCR contained only the
upstream priming oligonucleotide.   After 60 cycles of
amplification with an extension time of 9 min. the
product was removed from precursors by gel filtration.
35 Gel electrophoresis of the radioactive product showed a

major band corresponding in length to the 110 base
fragment. One quarter of this product (100,000 c.p.m.
in 0.9 M NaCl, TE, 0.1% SDS) was hybridised to the
array (a).   After 2 hours at 30° ca. 15000 c.p.m. had
been taken up. The melting behaviour of the hybrids
was followed as described for the 19-mer in example 3,
and it was found that the melting behaviour was similar
to that of the oligonucleotide.   That is to say, the
mismatches considerably reduced the melting temperature
of the hybrids, and conditions were readily found such
that the perfectly matched duplex remained whereas the
mismatched duplexes had fully melted.

Thus the invention can be used to analyse long
fragments of DNA as well oligonucleotides, and this
example shows how it may be used to test nucleic acid
sequences for mutations. In particular it shows how it
may be applied to the diagnosis of genetic diseases.

EXAMPLE 5.

To test an automated system for laying down the
precursors, the cosL oligonucleotide was synthesised
with 11 of the 12 bases added in the way described
above.   For the addition of the seventh base, however,
the slide was transferred into an Argon filled chamber
containing a pen plotter.   The pen of the plotter had
been replaced by a component, fabricated from Nylon,
which had the same shape and dimensions as the pen, but
which carried a polytetrafluoroethylene (PTFE) tube,
through which chemicals could be delivered to the
surface of the glass slide which lay on the bed of the
plotter.   A microcomputer was used to control the
plotter and the syringe pump which delivered the
chemicals.   The pen, carrying the delivery tube from
the syringe, was moved into position above the slide,
the pen was lowered and the pump activated to lay down
coupling solution. Filling the pen successively with

G, T and A phosphoramidite solutions an array of twelve
spots was laid down in three groups of four, with three
different oligonucleotide sequences. After
hybridisation to cosR, as described in Example 2, and

5 autoradiography, signal was seen only over the four
spots of perfectly matched oligonucleotides, where the
dG had been added.

EXAMPLE 6

This example demonstrates the technique of

10 analysing several DNA sequences simultaneously. Using
the technique described in Example 3, a slide was
prepared bearing six parallel rows of oligonucleotides
running along its length. These comprised duplicate
hexadecamer sequences corresponding to antisense

15 sequences of the β-globin wild-type (A), sickle cell
(S) and C mutations.

Clinical samples of AC, AS and SS DNA were
procured. Three different single-stranded probes of
110 nt length with approx. 70,000 c.p.m. in 100 μl 1M

20 NaCl, TE pH 7.5, 0.1% SDS, viz AC, AS, and SS DNA were
prepared. Radiolabelled nucleotide was included in the
standard PCR step yielding a double-stranded labelled
fragment. It was made single-stranded with
Bacteriophage λ exonuclease that allowed to selectively

25 digest one strand bearing a 5´ phosphate. This was
made possible by phosphorylating the downstream primer
with T4 Polynucleotide kinase and (´cold´) ATP prior to
PCR. These three probes were applied as three stripes
orthogonal to the surface carrying the six

30 oligonucleotide stripes. Incubation was at 30´C for 2
hours in a moist chamber. The slide was then rinsed at
ambient temperature, then 45´C for 5 minutes and
exposed for 4 days with intensification. The genotype
of each clinical sample was readily determined from the

35 autoradiographic signals at the points of intersection.

EXAMPLE 7

A plate was prepared whose surface carried an array of all 256 octapurines. That is to say, the array comprised 256 oligonucleotides each consisting

5 of a different sequence of A and G nucleotides. This array was probed with a mixture comprising all 256 octapyrimidines, each end labelled by means of polynucleotide kinase and γ-^{32}P-ATP. Hybridisation was performed for 6 - 8 hours at 4°C.

10 In consecutive experiments the hybridisation solvent was changed through the series 1M NaCl (containing 10mM Tris.HCl pH 7.5, 1mM EDTA, 7% sarcosine) and 2M, 2.5M, 3M, 3.5M, 4M, 4.5M, 5M and 5.5M TMACl (all containing 50mM Tris.HCl pH 8.0, 2mM

15 EDTA, SDS at less than 0.04 mg/ml). The plate was rinsed for 10 minutes at 4°C in the respective solvent to remove only loosely matched molecules, sealed in a plastic bag and exposed to a PhorphorImager storage phosphor screen at 4°C overnight in the dark.

20 The following table quotes relative signal intensities, at a given salt concentration, of hybrids formed with oligonucleotides of varying a content. In this table, the first row refers to the oligonucleotide GGGGGGGG, and the last row to the oligonucleotide

25 AAAAAAAA. It can be seen that the difference in response of these two oligonucleotides is marked in 1M NaCl, but much less marked in 3M or 4M TMACl.

30

35

Relative Intensities at given Salt Concentration

Solvent	Number of A's		
	0	4	8
1M NaCl	100	30	20
2M TMACl	100	70	30
3M TMACl	70	100	40
4M TMACl	60	100	40

The following table indicates relative signal intensities obtained, with octamers containing 4A's and 4G's, at different hybridisation salt concentrations. It can be seen that the signal intensity is dramatically increased at higher concentrations of TMACl.

Relative Intensities at different Salt Concentrations

Solvent	Yield of hybrid
1M NaCl	100
2M TMACl	200
3M TMACl	700
4M TMACl	2000

In conclusion, we have demonstrated the following:
1. It is possible to synthesise oligonucleotides in good yield on a flat glass plate.

2. Multiple sequences can be synthesised on the sample in small spots, at high density, by a simple manual procedure, or automatically using a computer controlled device.

5   3. Hybridisation to the oligonucleotides on the plate can be carried out by a very simple procedure. Hybridisation is efficient, and hybrids can be detected by a short autoradiographic exposure.

4. Hybridisation is specific. There is no detectable
10 signal on areas of the plate where there are no oligonucleotides. We have tested the effects of mismatched bases, and found that a single mismatched base at any position in oligonucleotides ranging in length from 12-mer to 19-mer reduces the stability of the
15 hybrid sufficiently that the signal can be reduced to a very low level, while retaining significant hybridisation to the perfectly matched hybrid.

5. The oligonucleotides are stably bound to the glass and plates can be used for hybridisation repeatedly.

20   The invention thus provides a novel way of analysing nucleotide sequences, which should find a wide range of application. We list a number of potential applications below:

25

30

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-27-

Small arrays of oligonucleotides as fingerprinting and mapping tools

Analysis of known mutations including genetic diseases.

Example 4 above shows how the invention may be
used to analyse mutations.   There are many
applications for such a method, including the detection
of inherited diseases.

Genomic fingerprinting.

In the same way as mutations which lead to disease
can be detected, the method could be used to detect
point mutations in any stretch of DNA.   Sequences are
now available for a number of regions containing the
base differences which lead to restriction fragment
length polymorphisms (RFLPs).   An array of oligo-
nucleotides representing such polymorphisms could be
made from pairs of oligonucleotides representing the
two allelic restriction sites.   Amplification of the
sequence containing the RFLP, followed by hybridisation
to the plate, would show which alleles were present in
the test genome.   The number of oligonucleotides that
could be analysed in a single analysis could be quite
large.   Fifty pairs made from selected alleles would
be enough to give a fingerprint unique to an individual.

Linkage analysis.

Applying the method described in the last
paragraph to a pedigree would pinpoint recombinations.
Each pair of spots in the array would give the
information that is seen in the track of the RFLP
analysis, using gel electrophoresis and blotting, that
is now routinely used for linkage studies.   It should
be possible to analyse many alleles in a single
analysis, by hybridisation to an array of allelic pairs
of oligonucleotides, greatly simplifying the methods
used to find linkage between a DNA polymorphism and
phenotypic marker such as a disease gene.

The examples above could be carried out using the

method we have developed and confirmed by experiments.

Large arrays of oligonucleotides as sequence reading tools.

We have shown that oligonucleotides can be
5   synthesised in small patches in precisely determined
positions by one of two methods: by delivering the
precursors through the pen of a pen-plotter, or by
masking areas with silicone rubber.   It is obvious how
a pen plotter could be adapted to synthesise large
10 arrays with a different sequence in each position.   For
some applications the array should be a predetermined,
limited set; for other applications, the array should
comprise every sequence of a predetermined length.   The
masking method can be used for the latter by laying
15 down the precursors in a mask which produces
intersecting lines.   There are many ways in which this
can be done and we give one example for illustration:
1.   The first four bases, A, C, G, T, are laid in four
broad stripes on a square plate.
20 2.   The second set is laid down in four stripes equal
in width to the first, and orthogonal to them.   The
array is now composed of all sixteen dinucleotides.
3.   The third and fourth layers are laid down in four
sets of four stripes one quarter the width of the first
25 stripes.   Each set of four narrow stripes runs within
one of the broader stripes.   The array is now composed
of all 256 tetranucleotides.
4.   The process is repeated, each time laying down two
layers with stripes which are one quarter the width of
30 the previous two layers.   Each layer added increases
the length of the oligonucleotides by one base, and the
number of different oligonucleotide sequences by a
factor of four.

The dimensions of such arrays are determined by
35 the width of the stripes.   The narrowest stripe we

have laid is 1mm, but this is clearly not the lowest
limit.

    There are useful applications for arrays in which
part of the sequence is predetermined and part made up
of all possible sequences. For example:

    Characterising mRNA populations.

    Most mRNAs in higher eukaryotes have the sequence
AAUAAA close to the 3' end.   The array used to analyse
mRNAs would have this sequence all over the plate. To
analyse a mRNA population it would be hybridised to an
array composed of all sequences of the type N_m AATAAAN$_n$.
For m + n = 8, which should be enough to give a unique
oligonucleotide address to most of the several thousand
mRNAs that is estimated to be present in a source such
as a mammalian cell, the array would be 256 elements
square.   The 256 x 256 elements would be laid on the
AATAAA using the masking method described above.   With
stripes of around 1mm, the array would be ca. 256mm
square.

    This analysis would measure the complexity of the
mRNA population and could be used as a basis for
comparing populations from different cell types.   The
advantage of this approach is that the differences in
the hybridisation pattern would provide the sequence of
oligonucleotides that could be used as probes to
isolate all the mRNAs that differed in the populations.

    Sequence determination.

    To extend the idea to determine unkown sequences,
using an array composed of all possible oligonucleotides
of a chosen length, requires larger arrays than we have
synthesised to date. However, it is possible to scale
down the size of spot and scale up the numbers to those
required by extending the methods we have developed and
tested on small arrays.   Our experience shows that the
method is much simpler in operation than the gel based
methods.

TABLE 1

For Examples 3 and 4 array (a) was set out as follows:

```
20  GAG GAC TCC TCT ACG          20  GAG GAC aCC TCT ACG
36  GAG GAC TCC TCT GAC G         20  GAC GAC aCC TCT GAC G
36  GAG GAC TCC TCT AGA CG        20  GAC GAC aCC TCT AGA CG
47  GAG GAC TCC TCT CAG ACG       36  GAG GAC aCC TCT CAG ACG
60  GAG GAC TCC TCT TCA GAC G     47  GAG GAC aCC TCT TCA GAC G
56  .AG GAC TCC TCT TCA GAC G     42  .AG GAC aCC TCT TCA GAC G
56  ..G GAC TCC TCT TCA GAC G     42  ..G GAC aCC TCT TCA GAC G
47  ... GAC TCC TCT TCA GAC G     42  ... GAC aCC TCT TCA GAC G
42  ... .AC TCC TCT TCA GAC G     36  ... .AC aCC TCT TCA GAC G
36  ... ..C TCC TCT TCA GAC G     36  ... ..C aCC TCT TCA GAC G
36  ... ... TCC TCT TCA GAC G     36  ... ... aCC TCT TCA GAC G
36  ... ... .CC TCT TCA GAC G     36  ... ... .CC TCT TCA GAC G
```

For example 3 array (b) was set out as follows:

```
20 GAG GAt TC            20 GAG GAC TC              20 GAG GAC aC
20 GAG GAt TCC           20 GAG GAC TCC             20 GAG GAC aCC
20 GAG GAt TCC T         20 GAG GAC TCC T           20 GAG GAC aCC T
20 GAG GAt TCC TC        20 GAG GAC TCC TC          20 GAG GAC aCC TC
20 GAG GAt TCC TCT       20 GAG GAC TCC TCT         20 GAG GAC aCC TCT
20 GAG GAt TCC TCT T     20 GAG GAC TCC TCT T       20 GAG GAC aCC TCT T
20 GAG GAt TCC TCT TC    20 GAG GAC TCC TCT TC      20 GAG GAC aCC TCT TC
20 GAG GAt TCC TCT TCA   20 GAG GAC TCC TCT TCA     20 GAG GAC aCC TCT TCA
32 GAG GAt TCC TCT TCA G    42 GAG GAC TCC TCT TCA G     20 GAG GAC aCC TCT TCA G
32 GAG GAt TCC TCT TCA GA   47 GAG GAC TCC TCT TCA G     32 GAG GAC aCC TCT TCA GA
42 GAG GAt TCC TCT TCA GAC  52 GAG GAC TCC TCT TCA GAC   42 GAG GAC aCC TCT TCA GAC
52 GAG GAt TCC TCT TCA GAC G 60 GAG GAC TCC TCT TCA GAC G 52 GAG GAC aCC TCT TCA GAC G
42 .AG GAt TCC TCT TCA GAC G 52 .AG GAC TCC TCT TCA GAC G 42 .AG GAC aCC TCT TCA GAC G
42 ..G GAt TCC TCT TCA GAC G 52 ..G GAC TCC TCT TCA GAC G 42 ..G GAC aCC TCT TCA GAC G
37 ... GAt TCC TCT TCA GAC G 47 ... GAC TCC TCT TCA GAC G 37 ... GAC aCC TCT TCA GAC G
32 ... .At TCC TCT TCA GAC G 42 ... .AC TCC TCT TCA GAC G 32 ... .AC aCC TCT TCA GAC G
32 ... ..t TCC TCT TCA GAC G 42 ... ..C TCC TCT TCA GAC G 32 ... ..C aCC TCT TCA GAC G
32 ... ... TCC TCT TCA GAC G 32 ... ... TCC TCT TCA GAC G 32 ... ... aCC TCT TCA GAC G
```

Between the three columns of array (b) listed above, were two columns, in which bases 6 and 7 from the left were missing in every line. These sequences all melted at 20 or 32 degrees. (a,t) mismatch base (.) missing base.